

APPENDIX A

Document No. AA, Morris, M.C., P. Vidal, L. Chaloin, F. Heitz and G. Divita. 1997. A new peptide vector for efficient delivery of oligonucleotides into mammalian cells. Nucl. Acids Res. 25 (No. 14): 2730-2735, discloses mixing of "MPG" with "oligonucleotides" as follows: namely,

"The development of antisense and gene therapy has focused mainly on improving methods for oligonucleotide and gene delivery into cells. In the present work, we describe a potent new strategy for oligonucleotide delivery based on the use of a short peptide vector, termed MPG (27 residues), which contains a hydrophobic domain derived from the fusion sequence of HIV gp41 and a hydrophilic sequence of SV40 T-antigen." (Abstract, page 2730, left column lines 1-10);

"Based on both intrinsic and extrinsic fluorescence titrations, it appears that the main binding between MPG and oligonucleotides occurs through electrostatic interactions which involve the basic-residues of the peptide vector. Further peptide/peptide interactions also occur, leading to a higher MPG/oligonucleotide ratio (in the region of 20/1), which suggests that oligonucleotides are most likely coated with several molecules of MPG. Premixed complexes of peptide vector with single or double stranded oligonucleotides are delivered into cultured mammalian cells in less than 1 h with relatively high efficiency (90%). This new strategy of oligonucleotide delivery into cultured cells based on a peptide vector offers several advantages compared to other commonly used approaches of delivery including efficiency, stability and absence of toxicity." The interaction with MPG strongly increases both the stability of the oligonucleotide to nuclease and crossing of the plasma membrane. The mechanism of cell delivery of oligonucleotides by MPG does not follow the endosomal pathway, which explains the rapid and efficient delivery of oligonucleotides in the nucleus. As such, we propose this peptide vector as a powerful tool for potential development in gene and antisense therapy." (Abstract, page 2730, lines 15-end of left column);

In the "Results and Discussion" section starting on page 2731, right column, bottom of page, appears the following description of MPG: namely,

"MPG was designed as a 27 residue peptide 'G-A-L-F-L-G-F-L-G-A-A-G-S-T-M-G-A-W-S-Q-P-K-S-K-R-K-V' with a cysteamide group at the C-terminus and an acetyl group at the N-terminus so as to improve its ability to cross the cell membrane (17). MPG is constituted of two independent domains linked together by a short peptide sequence: (i) a hydrophobic N-terminal domain (residues 1-17: G-A-L-F-L-G-F-L-G-A-A-G-S-T-M-G-A), the sequence of which derives from the glycine-rich region of the fusion sequence of viral gp41 (26, 27), known to be essential both for its membrane fusion activity and structural stabilization (26-28), and (ii) a hydrophilic C-terminal domain (residues 21-27: P-K-S-K-R-K-V) which derives from the nuclear localization signal of SV40 large T antigen, and which is potentially useful for improving nuclear addressing of the peptide (29,30)." (page 2731, bottom right column, last line- through page 2732, left column end of first paragraph);

At page 2733 in the "Results and Discussion" section appears a description of oligonucleotide stabilization during 2 hrs. nuclease digestion and 10 hrs. in serum containing medium as achieved using MPG: namely,

"One important problem in the delivery of oligonucleotides into cells is their high sensitivity to nucleases and low stability in cell culture medium supplemented with serum. The impact of the interaction of MPG with oligonucleotide was investigated by following the degradation of oligonucleotide by nuclease in the presence or not of MPG." (page 2733, right column, first 3 lines of text appearing under the heading "MPG improves the stability of oligonucleotides in cell culture medium supplemented with serum");

"As shown in Figure 4, the 18mer radiolabelled oligonucleotide is stable in the absence of serum (lane 2) and rapidly degraded by either DNase (lane 3) or serum (lane 4). In contrast, when oligonucleotide is preincubated with MPG before adding DNase (lane 5), no significant degradation is observed, suggesting that MPG strongly protects the oligonucleotide. MPG also protected the oligonucleotide from the nuclease in the cell culture medium supplemented with serum (lane 6)." (page 2733, right column, last paragraph, starting at line 10 of the paragraph and continuing to line 18);

At page 2735, under the heading "Conclusions" appears the following: namely,

"In the present work we have described a new strategy for oligonucleotide delivery into mammalian fibroblastic cells. This strategy is based on the use of a peptide vector, MPG, which exhibits relatively high affinity for single- and double-stranded oligonucleotides. The MPG peptide vector was designed by combining the fusion sequence of HIV gp41 (26,28) and the nuclear localization sequence of SV40 T antigen (29,30). When MPG is mixed with oligonucleotide in solution, they rapidly associate into a complex with tight non-covalent interactions. By simply overlaying this MPG-oligonucleotide complex onto mammalian cells, efficient delivery of the complex into the nucleus of the cells occurs in < 1 h." (page 2735, left column starting at the 1st line of the "Conclusions" section and continuing to the right column, line 5).

Table A, below, compares the sequence of MPG in Document AA with SEQ. ID. NO. 3 in United States Patent Application Serial No. 09/516,310. As disclosed therein, Residues 1-17 are "the sequence of which derives from the glycine-rich region of the fusion sequence of viral gp41", i.e., an importation competent signal peptide; and, Residues 18-27 are "the nuclear localization signal of SV40 large T antigen", i.e., a nuclear localization peptide.

TABLE A

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
MPG	G	A	L	F	L	G	F	L	G	A	A	G	S	T	M	G	A
Seq. ID No. 3		A	A	V	A	L	L	P	A	V	L	L	A	L	L	A	P
		*	†	†	†	†			†	†	†	†				†	†

18	19	20	21	22	23	24	25	26	27
W	S	Q	P	K	S	K	R	K	V
A	A	A	N	Y	K	K	P	K	L
					*	*		†	

*. Identical amino acids; †, conservative substitutions of one hydrophobic amino acid for another, Shaded, Positive charged residues

Document AB, Morris, M.C., L. Chaloin, J. Mery, F. Heitz and G. Divita. 1999. A novel potent strategy for gene delivery using a single peptide vector as a carrier. Nucl. Acids Res. 27 (No. 17): 3510-3517, discloses the following: namely,

"We have shown previously that a peptide, MPG derived from the hydrophobic fusion peptide of HIV-1 gp41 and the hydrophilic nuclear localisation sequence of SV40 large T antigen, can be used as a powerful tool for the delivery of oligonucleotides into cultured cells. Now we extend the potential of MPG to the delivery of nucleic acids into cultured cells. In vitro, MPG interacts strongly with nucleic acids, most likely forming a peptide cage around them, which stabilises and protects them from degradation in cell culture media. MPG is non-cytotoxic, insensitive to serum and efficiently delivers plasmids into several different cell lines in only 1 h. Moreover, MPG enables complete expression of the gene products encoded by the plasmids it delivers into cultured cells. Finally, we have investigated the potential of MPG as an efficient delivery agent for gene therapy, by attempting to deliver antisense nucleic acids targeting an essential cell cycle gene. MPG efficiently delivered a plasmid expressing the full-length antisense cDNA of human cdc25C, which consequently successfully reduced cdc25C expression levels and promoted a block to cell cycle progression. Based on our results, we conclude that MPG is a potent delivery agent for the generalized delivery of nucleic acids as well as of oligonucleotides into cultured cells and believe that its contribution to the development of new gene therapy strategies could be of prime interest." (Abstract, page 3510).

Document AC, Morris, M.C., L. Chaloin, F. Heitz and G. Divita. 2000. Translocating peptides and proteins and their use for gene delivery. *Current Opin. Biotech.* 11: 461-466, reportedly reviews the literature of translocating peptides and their uses for gene therapy and discloses the following: namely,

"The NLS" {nuclear localization signal} "from the large tumor antigen of Simian virus 40 (PKKKRKV) is one" {of} "the most used in non-viral gene delivery systems [35,39,40]. Peptides or proteins containing NLS, whether complexed to lipids or not, are used to improve nuclear transport through electrostatic binding of plasmids [8]. The non-covalent attachment of NLS to DNS is preferable as this enables complete release of DNA from the NLS peptide after nucleocytoplasmic transport. In the case of chimeric proteins containing a DNA-binding domain and an NLS, competition between these two moieties for DNA binding is prevented, and the accessibility of the NLS enhanced, by using neutral or anionic NLSs, or a longer NLS sequence with negatively charged residues upstream or downstream. A second approach is the covalent attachment of signal peptides to plasmid DNA to improve its nuclear localization [41-43]. " (page 463, left column, lines 22-37); and,

"An interesting alternative is the design of peptide-based gene delivery systems that bypass the endosomal pathway or that fuse with the plasma membrane at neutral pH. Our group has developed a single-chain peptide vector, MPG (GALFLGFLGAAGSTMGAWSQPKSKRKV), that associates a fusion peptide sequence derived from the hydrophobic fusion peptide of HIV-1 gp41 and the hydrophilic NLS of Simian virus 40 large T antigen. MPG can efficiently deliver short oligonucleotides and large plasmids into a wide variety of cell lines independently of the endosomal pathway [35,36]. This peptide interacts strongly with nucleic acids through its NLS domain, and forms peptide-peptide interactions through the gp41 hydrophobic domain, thus generating a peptide cage around the plasmid. After crossing the cell membrane, the presence of the NLS domain promotes fast delivery of the plasmid into the nucleus." (page 464, left column, lines 31-48).

Document AD, Morris, M.C., V. Robert-Hebmann, L. Chaloin, J. Mery, F. Heitz, C. Devaux, R.S. Goody and G. Divita. 1999. A New Potent HIV-1 Reverse Transcriptase Inhibitor. *J. Biol. Chem.* 274 (No. 35): 24941-24946, discloses the following at page 24943: namely,

"Cell Delivery of the Peptide Inhibitor - We further investigated the delivery of peptide p7 into cells and its antiviral activity on infected cell lines. The main problem in the delivery of drugs

into cells are crossing the cell membrane and reaching the target within the cell. To locate peptides in different cell lines, we exploited the inherent properties of the cysteamide group at the C-terminal end of the peptide to covalently link a fluorescent probe, Lucifer yellow. Peptide 7 was applied onto cultured human adherent HS-68 fibroblasts in the presence of 10% serum. In these conditions no degradation of the peptides could be detected after 1 h incubation. As shown in Fig. 3, peptide inhibitors entered cell poorly and after 1 h of incubation, localized in the cytoplasm (Fig. 3b). To overcome the lack of efficient cell delivery, we used a carrier peptidyl system (MPG) derived from the fusion peptide of gp41 and containing the nuclear localization sequence of SV40 large T antigen (22). This bifunctional carrier contains a hydrophobic N-terminal domain and a hydrophilic C-terminal moiety, the latter being extremely powerful for the delivery of oligonucleotides and plasmids into cells (22)." (page 24943, right column, lines 32-51);

"Binding of p7 to MPG was determined by measuring changes in the intrinsic Trp fluorescence of the peptide, upon titration of a fixed concentration of MPG (1 μ M) with increasing concentrations of p7. The corresponding titration curve (Fig. 3e), reveals that p7 interacts strongly with MPG, induces an important quenching of fluorescence up to 30%, with a dissociation constant of approximately 30 \pm 7nM. " (page 24943, right column, lines 54-60);

"The preformed MPG-p7 complex was separated and purified by size exclusion HPLC in a high concentration of salt (200mM NaCl). Three different subpopulations were observed (Fig. 3f): the main peak (1) corresponding to a molecular mass of ~50 kDa, which can be assessed as a complex of p7-MPG at a 1/20 ratio, when taking into account the molecular weight of each peptide (2.4 kDa for MPG and 0.8 kDa for p7); peaks 2 and 3 corresponding to lower molecular weight complexes (20 kDa) containing both MPG and p7 and to the monomeric form of MPG, respectively." (page 24944, bottom of page, right column, last line through page 24945, right column, line 6);

"The high molecular weight p7-MPG complex was purified and both its cellular localization and antiviral activity were analyzed. When complexed at a 20/1 ratio with MPG, p7 localized rapidly in the cytoplasm in less than 5 min, but after 30 min could be found mainly in the nucleus (Fig. 3, c and d). In contrast when complexed with MPG at a 10/1 ratio, most of p7 was retained in the cell membranes, suggesting that this complex is not stable enough in the cell culture medium. Hence, the formation of large particle seems to improve the stability of p7 and to increase its delivery into cells." (page 24945, right column, lines 16-25); and,

"Antiviral Activity of the Peptide Inhibitor - The ability of p7 and of the p7-MPG complex to inhibit HIV-1 infection in cultured CEM-T cells was measured by monitoring RT activity in the cell free culture supernatants. The peptides were added after a 30-min adsorption of the virus at 4°C, just before incubation of the cells at 37°C. Kinetics of HIV-1 production in the presence of p7 or MPG-p7 are reported in Fig. 5. We investigated the kinetics of viral production in the presence of different concentrations of p7 and MPG/p7, in comparison with two other HIV inhibitors: azidothymidine (10 μ M) and anti-CD4 antibody 13B8.2 (66nM)(28). In the presence of 100nM of p7 no virus was detected up to 22 days post-infection (Fig. 5a). For a concentration of 10nM of p7, viral replication was inhibited during the first 15 days, after which slow propagation of virus was detected in the RT assay and confirmed by dosage of p24 (data not shown). ...When p7 was complexed to MPG, antiviral activity was markedly improved, as no virus was detected 22 days after infection with 10 nM of p7 (Fig. 5b). Even at the lowest concentration used (0.1nM), no virus was detected up to 15 days post-infection." (page 24945, left column, line 40 through page 24946, left column, line 19).

Document AE, Biochemicals Organic Compounds for Research and Diagnostic Reagents. 1994. SIGMA Chemical Company. p. 295, shows the availability of at least 39 cross-linking coupling reagents at the time the priority application was filed.

Document AF, Rojas, M., J.P. Donahue, Z. Tan and Y-Z. Lin. 1998. Genetic engineering of proteins with cell membrane permeability. *Nature Biotech.* 16: 370, disclose as follows: namely,

"To determine if a similar MTS could mediate the cellular import of an entire protein, we genetically engineered *Schistosoma japonicum* glutathione S-transferase (GST; EC2.5.1.18) to contain a 12-residue MTS at its C-terminus. We show that the GST-MTS fusion protein, expressed in *Escherichia coli* and purified by glutathione-agarose affinity chromatography, was efficiently imported into living cells. When the Grb2 SH2 domain was fused to GST-MTS, the resulting 41-kDa GST-Grb2SH2-MTS protein was imported into cells and inhibited EGF-induced EGFR-Grb2 association and MAP kinase activation." (page 370, left column, lines 22-31).

Document AG, Murray, R.K., D. Granner, P.A. Mayes and V.W. Rodwell. 1993-1994. *Harper's Biochemistry*, Twenty Second Edition. Appleton & Lange, Norwalk, CT. p. 38., contained the following: namely,

"The initial achievements of the Merrifield technique" (1986) "were the synthesis of the A chain (21 residues) and B chain (30 residues) of insulin in 11 days and of the enzyme pancreatic ribonuclease in 18% overall yield. Subsequent improvements have reduced the time for synthesis of a peptide bond to about 1 hour and have increased yields significantly. This has initiated new prospects, not only for confirming de novo synthesis of the primary structures of proteins, but for immunology, for producing vaccines and polypeptide hormones, and conceivably also for treating selected inborn errors of metabolism." (page 38, right column, last paragraph before "References".)

Document AH, Liu, X.Y., D. Robinson, R.A. Veach, D. Liu, S. Timmons, R.D. Collins and J. Hawiger. 2000. Peptide-directed Suppression of a Pro-inflammatory Cytokine Response. *J. Biol. Chem.* 275 (22): 16774-16778, disclose as follows: namely,

"Signal-dependent nuclear translocation of transcription factor nuclear factor κ B (NF- κ B) is required for activation of downstream target genes encoding the mediators of immune and inflammatory responses. To inhibit this inducible signaling to the nucleus, we designed a cyclic peptide (cSN50) containing a cell-permeable motif and a cyclized form of the nuclear localization sequence for the p50-NF- κ B1 subunit of NF- κ B. When delivered into culture macrophages treated with the pro-inflammatory agonist lipopolysaccharide, cSN50 was a more efficient inhibitor of NF- κ B nuclear import than its linear analog. When delivered into mice challenged with lipopolysaccharide, cSN50 potently blocked the production of proinflammatory cytokines (tumor necrosis factor α and interferon γ) and significantly reduced the lethality associated with ensuing endotoxic shock. Based on specificity studies conducted with a mutated form of cSN50, a functional nuclear localization motif is required for this protective effect. Taken together, our findings demonstrate effective targeting of a cell-permeable peptide that attenuates cytokine signaling *in vivo*. This new class of biological response modifiers may be applicable to the control of systemic inflammatory reactions." (page 16774, abstract lines 1-24).

Document AI, Fujihara, S.M., J.S. Cleaveland, L.S. Grosmaire, K.K. Berry, K.A. Kennedy, J.J. Blake, J. Loy, B.M. Rankin, J.A. Ledbetter and S.G. Nadler. 2000. A D-amino acid peptide inhibitor of NF- κ B nuclear localization is efficacious in models of inflammatory disease. *J. Immunol.* 165: 1004-1012, disclose as follows: namely,

"The transcription factor NF- κ B regulates many genes involved in proinflammatory and immune responses. The transport of NF- κ B into the nucleus is essential for its biologic activity. We

describe a novel, potent, and selective NF- κ B inhibitor composed of a cell-permeable peptide carrying two nuclear localization sequences (NLS). This peptide blocks NF- κ B nuclear localization, resulting in inhibition of cell surface protein expression, cytokine production, and T cell proliferation. The peptide is efficacious in vivo in a mouse septic shock model as well as a mouse model of inflammatory bowel disease, demonstrating that NF- κ B nuclear import plays a role in these acute inflammatory disease models." (page 1004, abstract, lines 1-6); and,

"We synthesized a D-amino acid peptide (referred to as BMS 214572) containing a cell membrane-translocating sequence flanked by two nuclear localization sequences derived from *c-myc* (16,39)(Table 1)."

The amino acid sequence of residues 7-28 disclosed in Table 1 of Fugihara et al. is duplicated below in TABLE B and compared with SEQ.ID.NO.3 in Applicant instant application.

TABLE B*

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
BMS214572					V	A	A	V	A	L	L	P	A	V	L	L	A
Seq. ID No. 3		A	A	V	A	L	L	P	A	V	L	L	A	L	L	A	P
					†	†	†		*	†	*		*	†	*	†	

18	19	20	21	22	23	24	25	26	27
L	L	A	P	K	K	K	R	K	V
A	A	A	N	Y	K	K	P	K	L
†	†	*			*	*		*	†

*, Identical amino acids; †, conservative substitutions of one hydrophobic amino acid for another. Shaded. Positive charged residues

Document AJ, Schwarze, S.R., A. Ho, A. Vocero-Akbani and S.F. Dowdy. 1999. In Vivo Transduction: Delivery of a biologically active protein into mouse. Science 285: 1569-1572, disclose as follows: namely,

"Delivery of therapeutic proteins into tissues and across the blood-brain barrier is severely limited by the size and biochemical properties of the proteins. Here it is shown that intraperitoneal injection of the 120-kilodalton β -galactosidase protein, fused to the protein transduction domain from the human immunodeficiency virus TAT protein, results in delivery of the biologically active fusion protein to all tissues in mice, including the brain. These results open new possibilities for direct delivery of proteins into patients in the context of protein therapy, as well as for epigenetic experimentation with model organisms." (page 1569, abstract, lines 1-8); and,


"Synthetic TAT peptides contained an NH₂-terminal FITC-Gly residue that resulted in identical coupling rates between peptides [FITC-G-GGG-YGRKKRRQRRR (G, Gly; K, Lys; Q, Gln; R, Arg; Y, Tyr)]." (page 1572, under References and Notes, Note #7).

The amino acid sequence of TAT residues 1- disclosed in Note #7 of Schwarze et al. is duplicated below in TABLE C and compared with SEQ.ID.NO.3 in Applicant instant application.

TABLE C*

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
TAT													G	G	G	G	Y
Seq. ID No. 3		A	A	V	A	L	L	P	A	V	L	L	A	L	L	A	P
													†	†	†	†	

18	19	20	21	22	23	24	25	26	27
G	R	K	K	R	R	Q	R	R	R
A	A	A	N	Y	K	K	P	K	L

 +
*, Identical amino acids; †, conservative substitutions of one hydrophobic amino acid for another,
Shaded, Positive charged residues

Document AK, Xia, H., Q. Mao and B.L. Davidson. 2001. The HIV Tat protein transduction domain improves the biodistribution of b-glucuronidase expressed from recombinant viral vectors. *Nature Biotechnol.* 19: 640-644., discloses as follows: namely,

"Treatment of inherited genetic diseases of the brain remains an intractable problem. Methods to improve the distribution of enzymes that are injected or expressed from transduced cells will be required for many human brain therapies. Recent studies showed that a peptide, the protein transduction domain (PTD) from HIV Tat, could improve the distribution of cytoplasmic reporter proteins when administered systemically as fusion proteins or cross-linked chimeras. The utility of this motif for noncytoplasmic proteins has not been determined. Here, we tested how the Tat motif affected uptake and biodistribution of the lysosomal enzyme β -glucuronidase, the protein deficient in the disease mucopolysaccharidosis VII, when expressed from viral vectors. The Tat motif allowed for mannose-6-phosphate (M6P) independent uptake in vitro and significantly increased the distribution of β -glucuronidase secreted from transduced cells after intravenous or direct brain injection in mice or recombinant vectors. Thus, enzymes modified to contain protein transduction motifs may represent a general strategy for improving the distribution of secreted proteins following in vivo gene transfer." (page 640, abstract, lines 1-11).

Document AL, Lindsay, M.A. 2002. Peptide-mediated cell delivery: application in protein target validation. *Curr. Opinion in Pharmacol.* 2: 587-594, discloses as follows: namely,

"Interestingly, recent studies have identified several short peptide sequences named protein transduction domains (PTDs) or cell penetrating peptides (CPPs), which appear to rapidly translocate into all cells both in vitro and in vivo. Importantly, conjugation of proteins, peptides and antisense to these PTDs has been shown to deliver these cargoes effectively, allowing observation of biological action in several cell and animal models [1,2]. In this review, we examine the use of PTDs as a novel and potentially universal delivery system for delineation of protein function and target validation." (page 587, left column, Introduction section, lines 22-32).

Tabularized information in this document show more than 30 different intracellular targets effected in more than 40 different cell types (Table 1-2).